Characterization of the type III capsular polysaccharide produced by *Burkholderia pseudomallei*

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Burkholderia pseudomallei has been shown to produce more than one capsular polysaccharide (CPS). Analysis of the *B. pseudomallei* genome has revealed that the organism contains four CPS operons (I–IV). One of these operons (CPS III) was selected for further study. Comparative sequencing analysis revealed that the genes encoding CPS III are present in *B. pseudomallei* and *Burkholderia thailandensis* but not in *Burkholderia mallei*. In this study, CPS III was not found to contribute to the virulence of *B. pseudomallei*. Strains containing mutations in CPS III had the same LD₅₀ value as the wild-type when tested in an animal infection model. Production of CPS III was shown to be induced in water but inhibited in 30 % normal human serum using a *lux* reporter fusion assay. Microarray analysis of capsule gene expression in infected hamsters revealed that the genes encoding CPS III were not significantly expressed *in vivo* compared with the genes encoding the previously characterized mannoheptose capsule (CPS I), which is an important virulence factor in *B. pseudomallei*. Glycosyl-composition analysis by combined GC/MS indicated that the CPS III genes are involved in the synthesis of a capsule composed of galactose, glucose, mannose and xylose.

Received 16 May 2010

Accepted 12 August 2010

INTRODUCTION

Burkholderia pseudomallei is a Gram-negative bacillus and environmental saprophyte found in soil and stagnant waters in a number of regions around the world, particularly in areas that fall between 20° N and 20° S of the equator (Dance, 1991; Leelarasamee & Bovornkitti, 1989). The incidence of infection by this organism tends to increase during the rainy season when the water table rises, which results in the presence of more organisms at the surface (Currie & Jacups, 2003; Currie *et al.*, 2000a, 2004). Infection by *B. pseudomallei* is a result of either direct inoculation into wounds or inhalation of contaminated material (Leelarasamee & Bovornkitti, 1989). *B. pseudomallei* is the causative agent of melioidosis, a disease known as ‘the great mimicker’ because of its similarity to other infections and the difficulty in its diagnosis (Whitmore & Krishnaswami, 1912; Yee *et al.*, 1988). The clinical manifestations of melioidosis are wide, ranging from acute pneumonia or

acute septicaemia to chronic and latent infections, and the disease has an incubation period that ranges from 2 days to 63 years (Cheng & Currie, 2005; Ngauy *et al.*, 2005; Sanford, 1995). *B. pseudomallei* is inherently resistant to a number of antibiotics and, even with aggressive antibiotic therapy, the mortality rate remains high and the incidence of relapse is common (Currie *et al.*, 2000b; White *et al.*, 1989).

Capsules have been shown to contribute to the virulence of many bacterial pathogens (Boulnois & Roberts, 1990). Previously, our laboratory demonstrated that the capsular polysaccharide (CPS) with the structure [-3)-2-O-acetyl-6-deoxy- β -D-manno-heptopyranose-(1-)] was an important virulence determinant of *B. pseudomallei* (Reckseidler *et al.*, 2001). This capsule, which we designated CPS I, was found to be responsible for the survival of *B. pseudomallei* in the blood of the host by reducing opsonization and phagocytosis (Reckseidler-Zenteno *et al.*, 2005). A mutant in this capsule was also found to be attenuated in a mouse model of melioidosis (Atkins *et al.*, 2002). The same capsule is also produced by *Burkholderia mallei* and has been shown to be required for virulence in this organism (DeShazer *et al.*, 2001).

Sequence analysis of the completed genome of *B. pseudomallei* has revealed four operons with the predicted function

Abbreviations: CPS, capsular polysaccharide; Km, kanamycin; NHS, normal human serum; Pm, polymyxin B; RLU, relative light unit; Sm, streptomycin.

Further details of the virulence of the CPS III mutant in a hamster model, and of the glycosyl-composition and glycosyl-linkage analyses, are available as supplementary data with the online version of this paper.

of CPS biosynthesis and export (Holden *et al.*, 2004). One of these operons, CPS I, with the gene identifiers BPSS12786–BPSS12810, corresponds to the previously characterized mannoheptose capsule (Reckseidler *et al.*, 2001; Reckseidler-Zenteno *et al.*, 2005). Three other operons have been identified. One of these operons, with the gene identifiers BPSS0417–BPSS0429, was found to contain genes involved in the biosynthesis of a capsule with gene products that demonstrated homology to those of enteric capsule clusters and LPS biosynthesis operons. We designated this capsule locus CPS II. Another operon, with gene identifiers BPSS1825–BPSS1835, was also found to contain genes with predicted products involved in capsule biosynthesis and was designated CPS III. A fourth operon, CPS IV, with the gene identifiers BPSS12769–BPSS12785, was found to contain genes that may be involved in the synthesis of a capsule.

The objective of this work was to characterize one of these putative capsule operons and to determine the contribution of this capsule to virulence. The role of this capsule operon was recently investigated, but the aim of this study was to enhance the previous literature by performing comparative genomics among *Burkholderia* species, investigating gene expression patterns and virulence properties, and finally to determine the composition of this capsule (Sarkar-Tyson *et al.*, 2007).

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *B. pseudomallei* and *Escherichia coli* were grown at 37 °C on Luria–Bertani (LB) broth base (Becton Dickinson) agar plates or in LB broth. For animal studies, *B. pseudomallei* cultures were grown at 37 °C in TSBDC (tryptic soy broth dialyzed and chelated) medium (Brett *et al.*, 1997). Where appropriate, antibiotics were added at the following concentrations: 100 µg streptomycin (Sm) ml⁻¹, 25 µg gentamicin ml⁻¹, 25 µg kanamycin (Km) ml⁻¹ and 100 µg polymyxin B (Pm) ml⁻¹ for *B. pseudomallei*, and 100 µg ampicillin ml⁻¹, 25 µg gentamicin ml⁻¹ and 25 and 50 µg Km ml⁻¹ for *E. coli*.

DNA manipulation. Restriction enzymes and T4 DNA ligase were purchased from Invitrogen Life Technologies and New England Biolabs, and were used according to the manufacturers' instructions. Chromosomal DNA was isolated using a Wizard Genomic DNA purification kit (Promega). Plasmid DNA was isolated using a QIAprep spin miniprep kit (Qiagen). DNA fragments used in cloning procedures were excised from agarose gels and purified using a Qiagen gel extraction kit. PCR products were cloned into pCR2.1-TOPO using a TOPO TA cloning kit and chemically competent *E. coli* TOP10 (Invitrogen Life Technologies) according to the manufacturer's instructions.

Construction of lux reporter strain *B. pseudomallei* SZ1829. An internal fragment of the BPSS1829 (*bceG*) gene was amplified by PCR using primers 1829-int-F and 1829-int-R (Table 2). The DNA fragment was amplified from *B. pseudomallei* strain 1026b chromosomal DNA via PCR using a Qiagen HotStarTaq master mix kit as described previously (Moore *et al.*, 1999). PCR products were cloned into pCR2.1-TOPO according to the manufacturer's instructions. The 426 bp internal region from BPSS1829 was cloned as an *EcoRI* fragment into pGSV3-*lux*, resulting in the plasmid pSZ216 (Table 1).

TOP10(pSZ216) was conjugated with *B. pseudomallei* strain DD503 in a triparental mating protocol as described previously (Moore *et al.*, 1999; Reckseidler *et al.*, 2001).

Construction of capsule deletion mutants. To construct an in-frame deletion of the one of the CPS III genes, oligodeoxyribonucleotide primers were designed to amplify ~600 bp of DNA sequence upstream and downstream of the *wabG* gene, a predicted glycosyl-transferase with the gene identifier BPSS1826 (Table 3). The primers 1826-UP-*XbaI* and 1826-UP-*HindIII* (Table 2) were used to amplify the upstream DNA sequence (585 bp) including the ATG start site. The primers 1826-DOWN-*HindIII* and 1826-DOWN-*KpnI* were used to amplify a DNA fragment including the stop codon and downstream DNA. The primers 1826-UP-*HindIII* and 1826-DOWN-*HindIII* were designed to incorporate a unique *HindIII* restriction site to facilitate cloning of the upstream and downstream fragments to create an in-frame deletion of the *wbpY* gene. The primers 1826-UP-*XbaI* and 1826-DOWN-*KpnI* were designed to contain *XbaI* and *KpnI* linkers for cloning of the DNA fragments into the plasmids pBluescript SK and pKAS46 (Table 1). The DNA fragments were amplified from *B. pseudomallei* 1026b chromosomal DNA via PCR using a Qiagen HotStarTaq master mix kit as described previously (Reckseidler-Zenteno *et al.*, 2005) and cloned into pBluescript SK (Table 1). The assembled in-frame deletion was then cloned as an *XbaI/KpnI* fragment into pKAS46, an allelic-exchange vector based on *rpsL*, for counterselection, resulting in the plasmid pKAS46Δ1826 (Table 1). *B. pseudomallei* DD503 was the recipient strain used for allelic exchange (Table 1). Allelic exchange was performed as described previously, except that transconjugants were plated on Pm and Km (Moore *et al.*, 1999). The Pm^r Km^r transconjugants were subsequently transferred to plates containing Sm to select for the loss of pKAS46. Sm^r Km^s transconjugants were screened by PCR for the in-frame deletion using the primers 1826-UP-*XbaI* and 1826-DOWN-*KpnI*. The mutant strain *B. pseudomallei* DD503(Δ1826) contained the expected deletion in the *wabG* gene relative to wild-type 1026b.

A multiple deletion strain containing mutations in three of the characterized or predicted capsule operons (CPS I, II and IV) was constructed in order to create a strain that produced only CPS III. Deletions in one of the genes from the CPS II and IV operons were constructed as described above using the primers 418-UP-*XbaI*, 418-UP-*HindIII*, 418-DOWN-*HindIII* and 418-DOWN-*KpnI* to construct a deletion in the CPS II operon, and with the primers 2780-UP-*XbaI*, 2780-UP-*HindIII*, 2780-DOWN-*HindIII* and 2780-DOWN-*KpnI* to construct a deletion in the CPS IV operon (Table 2), resulting in the strains DD503(Δ418) and DD503(Δ2780) (Table 1). A CPS I deletion was constructed previously to create *B. pseudomallei* SZ210 (Table 1). SZ210, containing an in-frame deletion in the *wcbB* gene and deficient in CPS I production was used instead of DD503 for construction of the capsule triple-mutant strain. Plasmid pKAS46Δ418 was conjugated with SZ210 resulting in SZ210(Δ418), and plasmid pKAS46Δ2780 was conjugated with SZ210(Δ418) resulting in SZ210(Δ418)(Δ2780), a strain harbouring mutations in the CPS I, II and IV operons (Table 1). All deletions were confirmed by PCR as described above using the upstream and downstream primers for each gene of interest.

Animal studies. The animal model of acute *B. pseudomallei* infection has been described previously (DeShazer & Woods, 1999). To assess the virulence of the capsule mutant strain SZ1829 compared with wild-type *B. pseudomallei* 1026b, female Syrian hamsters aged 6–8 weeks (five per group) were inoculated intraperitoneally with 10¹, 10² or 10³ c.f.u. The control group (five hamsters) infected with wild-type 1026b were inoculated with 10¹ c.f.u. At 48 h, the numbers of hamsters that had succumbed were recorded, and the blood of infected animals was serially diluted and plated for bacterial quantification.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Reference/source
Strains		
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> ; Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>ara</i> Δ139 Δ(<i>ara-leu</i>)7697; <i>galU</i> <i>galK</i> <i>rpsL</i> (Sm ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ139 Δ(<i>ara-leu</i>); 7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i> λ ⁻ <i>tonA</i>	Invitrogen
HB101	F ⁻ Δ(<i>gpt-proA</i>)62 <i>leu</i> <i>supE44</i> <i>ara14</i> <i>galK2</i> <i>lacY1</i> ; Δ(<i>mcrC-mrr</i>) <i>rpsL20</i> (Sm ^r) <i>xyl-5</i> <i>mtl-1</i> <i>recA13</i>	Boyer & Roulland-Dussoix (1969)
S17-1λpir	Tp ^r Sm ^r <i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR-M</i> ⁺ RP4::2-Tc:Mu; Km Tn7 λpir	Biomedal Life Science
<i>B. pseudomallei</i>		
1026b	Clinical isolate; Km ^r Gm ^r Sm ^r Pm ^r Tc ^s Tp ^s	DeShazer <i>et al.</i> (1997)
DD503	1026b derivative; allelic exchange strain; Δ(<i>amrR-oprA</i>) (Km ^s Gm ^s Sm ^s) <i>rpsL</i> (Sm ^r)	Moore <i>et al.</i> (1999)
SR1015	1026b(pSR1015); Sm ^r Tc ^r	Reckseidler <i>et al.</i> (2001)
SZ210	DD503 derivative; Δ <i>wcbB</i>	Reckseidler-Zenteno <i>et al.</i> (2005)
SZ211	DD503 derivative; (pML102); Gm ^r	Reckseidler-Zenteno <i>et al.</i> (2005)
SZ1829	DD503 derivative; (pSZ216); Gm ^r	This study
DD503(Δ418)	DD503 derivative; (pKAS46Δ418); Δ <i>kpsE</i>	This study
DD503(Δ1826)	DD503 derivative; (pKAS46Δ1826); Δ <i>wabG</i>	This study
DD503(Δ2780)	DD503 derivative; (pKAS46Δ2780); Δ <i>wza</i>	This study
SZ210(Δ418)	SZ210 derivative; (pKAS46Δ418); Δ <i>wcbB</i> , Δ <i>kpsE</i>	This study
SZ210(Δ418)(Δ2780)	SZ210 derivative; (pKAS46Δ418), (pKAS46Δ2780); Δ <i>wcbB</i> , Δ <i>kpsE</i> , Δ <i>wza</i>	This study
<i>B. thailandensis</i>		
E264	Soil isolate; Km ^r Gm ^r Sm ^r Pm ^r Tc ^s Tp ^s	Brett <i>et al.</i> (1998)
Plasmids		
pGSV3- <i>lux</i>	Mobilizable suicide vector containing <i>lux</i> operon from <i>P. luminescens</i> ; OriT; Gm ^r	Moore <i>et al.</i> (2004)
pCR2.1-TOPO	Topoisomerase-mediated cloning vector; Ap ^r Km ^r	Invitrogen
pBluescript SK	General cloning vector; ColEI; Ap ^r	Stratagene
pKAS46	Mobilizable allelic exchange vector; π-dependent; R6K replicon; Ap ^r Km ^r <i>rpsL</i> ⁺ (Sm ^s)	Moore <i>et al.</i> (1999)
pRK2013	Self-transmissible helper plasmid; Km ^r	Figurski & Helsinki (1979)
pSZ216	426 bp internal region of CPS III <i>bceG</i> gene cloned as an <i>EcoRI</i> fragment into pGSV3- <i>lux</i> ; Gm ^r	This study
pKAS46Δ418	In-frame deletion of CPS II <i>kpsE</i> gene cloned as an <i>XbaI</i> - <i>KpnI</i> fragment into pKAS46; Ap ^r Km ^r	This study
pKAS46Δ1826	In-frame deletion of CPS III <i>wabG</i> gene cloned as an <i>XbaI</i> - <i>KpnI</i> fragment into pKAS46; Ap ^r Km ^r	This study
pKAS46Δ2780	In-frame deletion of CPS IV <i>wza</i> gene cloned as an <i>XbaI</i> - <i>KpnI</i> fragment into pKAS46; Ap ^r Km ^r	This study

Ap, Ampicillin; Gm, gentamicin; Tc, tetracycline; Tp, trimethoprim.

Western blot analysis of capsule production. Whole-cell extracts of *B. pseudomallei* strains for Western blot analysis were prepared as described previously (Bryan *et al.*, 1994). Immunoassay was performed with a 1:500 dilution of the primary antibody, mouse mAb 147 raised against *B. pseudomallei* CPS (Reckseidler-Zenteno *et al.*, 2005). The secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse polyvalent immunoglobulins (IgA, IgM and IgG; Sigma).

Sequence analysis of capsule clusters. Sequence analysis of the *B. pseudomallei*, *B. mallei* and *Burkholderia thailandensis* genomes was performed using the Artemis program (version 7) provided by the Sanger Centre (<http://www.sanger.ac.uk/Projects/>), the CMR BLAST program through The Institute for Genomic Research (<http://www.tigr.org/>) and using the BLAST program through the National Center for Biotechnology website (<http://www.ncbi.nlm.nih.gov/>).

Bioluminescence assay to investigate capsule regulation. Regulation of the *B. pseudomallei* capsule operons CPS I and III under a number of conditions was performed as described previously (Reckseidler *et al.*, 2001). The *lux* reporter strains *B. pseudomallei* SZ211 (CPS I⁻) and SZ1829 (CPS III⁻) were inoculated into LB broth, M9 medium plus 1 % glucose, M9 plus 1 % glucose plus 30 % normal human serum (NHS) or water. Ninety-six-well plates containing 150 μl medium were inoculated with 5 μl overnight culture of each strain (five replicates per condition) and incubated at 37 °C with shaking, except for the water samples, which were incubated at 37 °C without shaking to replicate the condition of

Table 2. Sequences of the primers used in this study

Primer	Sequence (5'→3')
1829-int-F	GCTCACGGTGTCTGGGCAAGAT
1829-int-R	GCTCAACACGACATGCACGAG
1826-UP- <i>Xba</i> I	GGGTCTAGAGCTTCATCTCGTTCTGTCTGT
1826-UP- <i>Hind</i> III	GATAAGCTTTGACGATGGCGATTCTCACTG
1826-DOWN- <i>Hind</i> III	GCGAAGCTTTGACGACGAGGCGGTCGTAAT
1826-DOWN- <i>Kpn</i> I	GCGGGTACCCAGATCGAATTGCGCGGTGTC
418-UP- <i>Xba</i> I	GGGTCTAGACCGCGCAGCAGGCGCAACT
418-UP- <i>Hind</i> III	GATAAGCTTGACGATGCGCGCGATCAG
418-DOWN- <i>Hind</i> III	GCGAAGCTTGTTCATCGGCAGGGCGTT
418-DOWN- <i>Kpn</i> I	GCGGGTACCTATCGAGCACGCCGAAGC
2780-UP- <i>Xba</i> I	GGGTCTAGATCCGTTCAAGTGCCTGAAGT
2780-UP- <i>Hind</i> III	GATAAGCTTACTACCCATCCACACCTCTAT
2780-DOWN- <i>Hind</i> III	GCGAAGCTTGTGACCAAATGATCGAATCGA
2780-DOWN- <i>Kpn</i> I	GCGGGTACCTATGGGGTCTTGGAGATTC

stagnant water. Optical density readings (OD₅₄₀) and luminescence measurements [in relative light units (RLU)] were taken every hour. The OD₅₄₀ values of the bacterial cultures were measured using a Bio-Rad microplate reader and Microplate Manager version 5.1 software (Bio-Rad). Luminescence was measured with a reporter microplate luminometer and reporter software (Turner Biosystems). Luminescence/absorbance was calculated to determine the level of gene expression for each strain and a difference in expression greater than 2.5-fold was considered significant.

Microarray analysis of capsule genes *in vivo* and *in vitro*.

Capsule gene expression *in vivo* was assessed using a previously described low-density DNA oligo-microarray (Moore *et al.*, 2004; Tuanyok *et al.*, 2005). Total RNA was isolated from the lungs and livers of *B. pseudomallei*-infected hamsters at 40 h and from *B. pseudomallei* grown in LB broth. For the *in vivo* data, five hamsters were injected with 100 c.f.u. *B. pseudomallei* 1026b. Serial dilutions and plate counts were used to confirm the dose. All hamsters were

severely ill at 40 h after the inoculation. This time point was chosen based on previous data generated from our laboratory indicating that the bacterial loads in the liver and lungs at 48 h from an inoculum of 100 c.f.u. is approximately 10⁴ c.f.u. ml⁻¹ (Reckseidler-Zenteno *et al.*, 2005). The 40 h time point was chosen instead of 48 h to ensure that the animals were still alive but close to death when sacrificed. At this time, all hamsters were euthanized, and the infected lungs and livers were preserved in RNAlater solution (Ambion) at -70 °C until required. For the *in vitro* results, bacterial cells were harvested from 20 ml culture in LB broth at an OD₆₀₀ 0.9. Prior to the harvesting of the cells, 1% (v/v) RNAlater was added to the culture, which was maintained at 4 °C. Following total bacterial RNA purification, bacterial mRNA purification was performed from both sources described above using MICROBExpress (Ambion) following the manufacturer's instructions. The level of gene expression *in vivo* was determined by comparing the *in vivo* and *in vitro* results. Scanning was performed with a Virtek ChipReader (Virtek Biotech) and the fluorescence signals from both dyes were quantified using QuantArray

Table 3. *B. pseudomallei* CPS III genes, homologous proteins and predicted functions

Gene identifier	Size (bp)	Homologous protein	Predicted protein function	Identity	Organism	Reference
BPSS1825	1167	HepB	Glycosyltransferase	37 %	<i>Anabaena</i> spp.	Wang <i>et al.</i> (2007)
BPSS1826	1185	WabG	Glycosyltransferase	29 %	<i>Klebsiella pneumoniae</i>	Regué <i>et al.</i> (2005)
BPSS1827	1476	CheA	Chemotaxis histidine kinase	35 %	<i>Pseudomonas fluorescens</i>	de Weert <i>et al.</i> (2002)
BPSS1828	1140	Cap1E	Glycosyltransferase	34 %	<i>Pseudomonas aeruginosa</i>	Raymond <i>et al.</i> (2002)
BPSS1829	948	BceG	Glycosyltransferase	77 %	<i>Burkholderia cepacia</i>	Moreira <i>et al.</i> (2003)
BPSS1830	2220	Wzc	Capsule export, tyrosine-protein kinase	36 %	<i>Escherichia coli</i>	Grangeasse <i>et al.</i> (2003)
BPSS1831	1176	Wza	Capsule export, outer-membrane protein	36 %	<i>Escherichia coli</i>	Dong <i>et al.</i> (2006)
BPSS1832	435	Wzb	Transport, tyrosine-protein phosphatase	40 %	<i>Escherichia coli</i>	Lescop <i>et al.</i> (2006)
BPSS1833	1422	RkpK	UDP-glucose-6-dehydrogenase	50 %	<i>Rhizobium meliloti</i>	Kereszt <i>et al.</i> (1998)
BPSS1834	1416	WcaJ	Sugar transferase	39 %	<i>Salmonella typhimurium</i>	Stevenson <i>et al.</i> (2000)
BPSS1835	1461	ManC	GDP-mannose-pyrophosphorylase	48 %	<i>Salmonella enterica</i>	Jensen & Reeves (2001)

microarray analysis software (Packard Bioscience). Normalization and data analysis were performed with GeneTraffic software (Iobion Informatics). Data obtained from at least 24 hybridizations of two independent RNA preparations of each type of infected organ including flip-dye replications were used in each analysis. Normalization of data was performed using LOWESS (locally weighted scatter plot smoother), a curve-fitting algorithm. To select significantly expressed genes, the following were used: differences (n -fold), mean log₂ ratio, SD, coefficient of variance and Student's t -test. Hierarchical clustering and K -means clustering analyses were performed using The Institute for Genomic Research MeV software (www.tigr.org/). The data are represented as the *in vivo* expression divided by the *in vitro* expression for all of the infected organs combined.

Purification of CPS III. Purification of CPS III was achieved by utilizing a multiple deletion strain containing mutations in the known and predicted capsule operons of *B. pseudomallei*, except for the CPS III operon. Capsule purification was performed on *B. pseudomallei* SZ210(Δ 418)(Δ 2780) according to a published hot phenol extraction procedure (Perry *et al.*, 1995; Westphal & Jann, 1965). The purified capsule was not delipidated.

Glycosyl-composition and glycosyl-linkage analysis of CPS III. Glycosyl-composition analysis was performed on the purified capsule by combined GC/MS. An aliquot of the sample was added to a glass tube with 20 μ g inositol as the internal standard. Methylglycosides were prepared from the dry sample following mild acid treatment by methanolysis in 1 M HCl in methanol at 80 °C for 16 h, followed by re- N -acetylation with pyridine and acetic anhydride in methanol (for the detection of amino sugars). The sample was then per- O -trimethylsilylated by treatment with Tri-Sil (Pierce) at 80 °C for 30 min. These procedures were carried out as described elsewhere (Merkle & Poppe, 1994; York *et al.*, 1986). GC/MS analysis of the trimethylsilylated methylglycosides was performed on an AT 6890N GC interfaced to a 5975B MSD, using a Supelco EC-1 fused silica capillary column (30 m $\times$ 0.25 mm internal diameter).

For glycosyl-linkage analysis, the sample was permethylated, depolymerized, reduced and acetylated. The resulting partially methylated alditol acetates were analysed by GC/MS as described by York *et al.* (1986).

RESULTS

B. pseudomallei contains four polysaccharide operons

Three of the identified capsule operons in the genome of *B. pseudomallei* were analysed further using the BLAST program and Artemis. The operons are illustrated in Fig. 1. The operon consisting of the genes BPSS0417–BPSS0429 was designated CPS II (Fig. 1a). Another operon, BPSS1825–BPSS1835, was designated CPS III and the predicted homologues were investigated further (Fig. 1b, Table 3). A fourth operon, CPS IV, was found to contain genes that may be involved in the synthesis of a capsule, with the gene identifiers BPSL2769–BPSL2785 (Fig. 1c).

Distribution of capsule loci among the three *Burkholderia* species

Comparative analysis of the genomes of *B. pseudomallei*, *B. mallei* and *B. thailandensis* was performed to determine

whether all of the predicted *B. pseudomallei* capsule operons were present in *B. mallei* and *B. thailandensis*. As shown in Fig. 1, all three capsules were found to be present in *B. pseudomallei* and *B. thailandensis* but not *B. mallei*. This is in contrast to CPS I, which is present in *B. pseudomallei* and *B. mallei* but not in *B. thailandensis* (DeShazer *et al.*, 2001; Holden *et al.*, 2004; Reckseidler *et al.*, 2001; Reckseidler-Zenteno *et al.*, 2005; Yu *et al.*, 2006). CPS II was found to be identical in *B. pseudomallei* and *B. thailandensis*, but *B. thailandensis* was found to contain two flanking hypothetical genes not present in *B. pseudomallei* (Fig. 1a). The CPS II genes were found to be deleted entirely from *B. mallei* (Fig. 1a). A large chromosomal region ranging from ORFs BPSS0404 to BPSS0491 including CPS II was shown to be deleted in *B. mallei* compared with *B. pseudomallei* and was replaced with a large chromosomal region containing ORFs BMAA0555 (IS407A *orfB*) to BMAA1784, a unique hypothetical protein not found in *B. pseudomallei* (Fig. 1a). The two genomes aligned with the presence of the alkyl hydroperoxidase reductase genes *ahpC* and *ahpF*, but these were organized in the opposite orientation in *B. mallei* compared with *B. pseudomallei* (Fig. 1a). The entire CPS III operon and flanking genes were shown to be the same in both *B. pseudomallei* and *B. thailandensis* (Fig. 1b). In contrast, the majority of the CPS III cluster was deleted from *B. mallei* with the exception of the *wcaJ* and *manC* genes, as well as the two flanking hypothetical genes on one side and another hypothetical gene on the other side of the deleted region (Fig. 1b). The deleted region of the CPS III genes in *B. mallei* was found to be replaced with the IS407A *orfA* and *orfB* genes (Fig. 1b). The entire CPS IV region was found to be replaced in *B. mallei* and flanked by two IS407A elements (Fig. 1c). The ORF BPSL2785, which encodes a hypothetical protein, was present in *B. mallei* (BMA2284.1) ATCC 23344, as well as in a number of other *B. mallei* strains, but was organized in the opposite orientation. The genomes of *B. pseudomallei*, *B. thailandensis* and *B. mallei* all diverged upstream of the CPS IV region, but all three organisms were found to align at the location of the *ompA* and hypothetical genes (Fig. 1c).

Mutation in CPS III does not affect the production of CPS I

Western blot analysis of CPS I production was performed using whole-cell extracts of strains containing mutations in the CPS I and III operons to confirm that a disruption in the CPS III cluster had no effect on CPS I production. As shown in Fig. 2, SZ1829, containing a mutation in CPS III, was shown to produce CPS I. This was demonstrated by the reaction to a mAb to CPS I, as indicated by the presence of a 200 kDa band. The reaction of this strain was similar to that of wild-type *B. pseudomallei* strains 1026b and DD503 and was in contrast to that of *B. pseudomallei* SZ210, which harboured a deletion in the CPS I operon and was deficient in CPS I production.

Fig. 1. Organization of the chromosomal regions containing CPS II, III and IV in *B. pseudomallei* K96243, *B. thailandensis* E264 and *B. mallei* ATCC 23344. The capsule operon and the genes involved are indicated in each figure. Gene names are shown according to homologous gene products as determined by the Artemis program and BLAST searches. Arrows indicate the direction of transcription. Genes identical among species are indicated in black and genes unique to a species are indicated in grey. (a) The chromosomal region containing CPS II and flanking DNA in *B. pseudomallei*, *B. thailandensis* and *B. mallei*. This region extends from nt 546190 to 671369 in *B. pseudomallei*, nt 2398500 to 2421308 in *B. thailandensis* and nt 563346 to 1957789 in *B. mallei*. (b) The chromosomal region containing CPS III and flanking DNA. This region extends from nt 2463970 to 2499713 in *B. pseudomallei*, nt 643144 to 676838 in *B. thailandensis* and nt 263152 to 270492 in *B. mallei*. (c) The chromosomal region containing CPS IV and flanking DNA. This region extends from nt 3184073 to 3199575 in *B. pseudomallei*, nt 1679130 to 1694403 in *B. thailandensis* and nt 2269377 to 2283058 in *B. mallei*. *hyp*, Hypothetical protein gene.

Fig. 2. Western blot analysis of CPS I production in *B. pseudomallei* SZ1829. Whole-cell extracts of wild-type and mutant strains of *B. pseudomallei* were prepared and reacted with a mAb to CPS I. The *B. pseudomallei* strains 1026b, DD503, SZ210 and SZ1829 are indicated.

CPS III does not contribute to virulence in the Syrian hamster model of melioidosis

To assess the role of CPS III in virulence, a strain with a mutation in the CPS III operon was tested for virulence compared with wild-type *B. pseudomallei* in the Syrian hamster model of melioidosis. Syrian golden hamsters were inoculated intraperitoneally with 10^1 – 10^3 cells of wild-type *B. pseudomallei* 1026b or the capsule mutant SZ1829. After 48 h, the LD₅₀ values were determined. As shown in Table 4, SZ1829 had LD₅₀ values of <10 c.f.u., identical to that of wild-type *B. pseudomallei*, indicating that this capsule is not required for virulence (see also Supplementary Table S1 available with the online journal). In addition, the bacterial load in the blood of the infected hamsters was similar to that of the wild-type strain, and was significantly higher than that of the non-pathogenic *B. thailandensis* E264 and the CPS I mutant *B. pseudomallei* SR1015, both of which are incapable

Table 4. Virulence of the CPS III mutant in the Syrian hamster model of acute melioidosis

Strain	CPS cluster inactivated	LD ₅₀ (c.f.u.)*	Blood bacterial count (c.f.u. ml ⁻¹)
1026b	None	<10	4.1×10^5
E264	None	6.8×10^5	0
SR1015	CPS I	3.5×10^5	3
SZ1829	CPS III	<10	1.9×10^5

*LD₅₀ values for 1026b, E264 and SR1015 were calculated previously (Brett *et al.*, 1997; Reckseidler *et al.*, 2001).

of establishing bacteraemia (Brett *et al.*, 1997; Reckseidler-Zenteno *et al.*, 2005). This indicated that CPS III does not contribute to persistence in the blood.

CPS III is not expressed in NHS

A *lux* reporter strain was constructed in the CPS III operon by cloning an internal fragment of one of the genes into pGSV3-*lux*, a suicide vector containing a promoterless *lux* operon from *Photobacterium luminescens* (Table 1). Regulation of this capsule in an environment similar to that encountered in the host was determined by growing the *lux* reporter strain SZ1829 in the presence of M9 plus 1% glucose versus M9 plus 1% glucose plus 30% NHS (Fig. 3). The OD₅₄₀ values and luminescence measurements were determined every hour. As shown in Fig. 3(b), expression of CPS III (SZ1829) was higher in M9 plus 1% glucose alone compared with M9 plus 1% glucose plus 30% NHS. The expression of SZ1829 was threefold to fourfold lower in 30% NHS. This was in contrast to the expression of CPS I (SZ211), which was significantly more highly expressed in 30% NHS at a level of threefold to fourfold compared with growth in M9 plus 1% glucose alone (Fig. 3a). Although CPS III demonstrated higher expression initially in 30% NHS, this may have been due to the fact that the addition of NHS caused precipitation in the medium, which affected the OD of the cultures.

CPS III is expressed in water

Expression of the *lux* operon in reporter strains SZ211 (CPS I⁺) and SZ1829 (CPS III⁺) was also measured in water to determine whether CPS III was induced in this environment. Overnight cultures of SZ211 and SZ1829 were inoculated into sterile water and incubated at 37 °C without shaking. Capsule expression was determined as described above, but the luminescence/absorbance calculations for water were compared with the values for these strains when grown in LB broth. CPS III was found to be induced in water compared with LB broth (Fig. 4b). The expression of SZ1829 was found to be significant, with an increase of twofold to threefold over the course of the experiment (Fig. 4b). The expression of CPS I was found to be more than fourfold higher in LB broth compared with water (Fig. 4a).

Microarray analysis of CPS III expression *in vivo*

Microarray analysis of capsule expression was performed using a low-density DNA microarray. RNA was isolated from the livers and lungs of hamsters infected with *B. pseudomallei* and from *B. pseudomallei* grown in LB broth. The results of the microarray experiment are shown in Table 5. The level of gene expression, or fold change, is represented as the ratio of gene expression in the hamster compared with growth in LB broth. As shown in Table 5, CPS III genes were not found to be significantly expressed *in vivo*, as most of the fold changes

Fig. 3. Expression of *B. pseudomallei* capsules CPS I and III in the presence of 30% NHS. *B. pseudomallei lux* reporter strains SZ211 (a) and SZ1829 (b) were grown in M9 plus 1% glucose or in M9 plus 1% glucose plus 30% NHS at 37 °C with shaking. The OD₅₄₀ and luminescence (RLU) were measured at each time point. Capsule expression was determined by normalizing light production against growth (RLU/OD₅₄₀) of each strain for each time point. The data represent the mean $\pm$ SD of *lux* reporter activity normalized for growth (five replicates per experiment) from three separate experiments. $\blacklozenge$, M9 + glucose; $\blacksquare$, M9 + NHS.

were determined to be less than twofold. Many of the genes had negative fold change values, indicating that these genes are suppressed in the host environment. The highest fold change result was 2.337866 for BPSS1827, a predicted glucose-6-phosphate isomerase, which was still much lower than the fold changes observed for CPS I genes, which were significantly higher (Tuanyok *et al.*, 2006).

Carbohydrate composition of CPS III

Glycosyl-composition analysis was performed on the purified capsule by combined GC/MS. The results indicated that CPS III is composed of galactose, glucose, mannose, xylose and rhamnose residues, with the highest proportion of carbohydrate being galactose and glucose (Table 6). Glycosyl-linkage

Fig. 4. Expression of *B. pseudomallei* capsules CPS I and III in the presence of water. *B. pseudomallei lux* reporter strains SZ211 (a) and SZ1829 (b) were inoculated into water and incubated at 37 °C. OD₅₄₀ and luminescence (RLU) were measured at each time point. Capsule expression was determined by normalizing light production against growth (RLU/OD₅₄₀) of each strain for each time point and comparing these results with growth in LB broth. The data represent the mean $\pm$ SD of *lux* reporter activity normalized for growth (five replicates per experiment) from three separate experiments. $\blacklozenge$, Water; $\blacksquare$, LB broth.

analysis was also performed (Table 7). The predominant glycosyl residue detected was a terminally linked heptopyranosyl at a percentage of 23.2. Other residues detected were a terminally linked and a 4-linked glucopyranosyl at percentages of 14.6 and 10.8, respectively. Supplementary Figs S1 and S2 (available with the online journal) illustrate the polysaccharide peaks for the glycosyl-composition and glycosyl-linkage analysis as determined by GC/MS.

DISCUSSION

Although significant advances have been made, melioidosis continues to be a public-health concern in many regions of the world (Cheng & Currie, 2005). Completion of the sequencing of the *B. pseudomallei* genome has revealed potential virulence determinants, and comparative genomics among the genomes of *B. pseudomallei*, *B. thailandensis* and *B. mallei* species has contributed to a better understanding of the organism. Further studies are

Table 5. Microarray analysis of *B. pseudomallei* CPS III expression following intraperitoneal inoculation in the hamster model of melioidosis

Gene identifier	Predicted protein function	Fold change (<i>in vivo</i> vs <i>in vitro</i>)*
BPSS1825	Glycosyltransferase	1.757155
BPSS1826	Glycosyltransferase	0.093565
BPSS1827	Glucose-6-phosphate isomerase	2.337866
BPSS1828	Glycosyltransferase	-0.66607
BPSS1829	Glycosyltransferase	-0.18061
BPSS1830	Capsule export, tyrosine-protein kinase	-0.30655
BPSS1831	Capsule export, outer-membrane protein	0.725849
BPSS1832	Transport, tyrosine-protein phosphatase	1.52665
BPSS1833	UDP-glucose-6-dehydrogenase	-0.71411
BPSS1834	Sugar transferase	-0.07963
BPSS1835	Mannose-1-phosphate guanylttransferase	-0.75075

*Gene expression representative of the liver and lungs of infected hamsters.

ongoing to define the pathogenesis of *B. pseudomallei* and to identify effective vaccine candidates and diagnostic targets (Cheng & Currie, 2005; Holden *et al.*, 2004).

Sequence analysis of the genome of *B. pseudomallei* revealed the presence of four operons possibly involved in CPS biosynthesis. One of these operons (CPS I) corresponded to the previously identified and characterized mannoheptose capsule that has been shown to be responsible for virulence and comprises one of the currently proposed melioidosis and glanders subunit conjugate vaccines (Brett & Woods, 1996, 2000; Reckseidler *et al.*, 2001; Reckseidler-Zenteno *et al.*, 2005; Warawa & Woods, 2002). The CPS I capsule cluster is also present in the genome of *B. mallei*, but the complete cluster is not found in the genome of *B. thailandensis* (DeShazer *et al.*, 2001; Nierman *et al.*, 2004; Yu *et al.*, 2006). This correlates with previous studies that

have shown that this capsule is produced by *B. mallei* but not by *B. thailandensis* (Brett *et al.*, 1997; DeShazer *et al.*, 1998, 2001; Reckseidler *et al.*, 2001).

Three other putative capsule operons were identified by sequence analysis, and all of these operons were found to be present in *B. pseudomallei* and *B. thailandensis* but not *B. mallei*. As these capsules are found in *B. thailandensis* and *B. pseudomallei*, they may be required for survival in either the host or the environment; however, further studies are required to determine the roles of CPS II and IV.

CPS III, located on chromosome 2, was found to contain 11 genes involved in the biosynthesis of a polysaccharide and was shown to be present in the genomes of *B. pseudomallei* and *B. thailandensis* but not *B. mallei*. A mutation in the CPS III cluster did not affect production of CPS I and so it can be concluded that this operon encodes

Table 6. Glycosyl composition of CPS III following analysis by GC/MS

Glycosyl residue	Mass (μg)	Mol%
Arabinose	ND	–
Rhamnose	0.5	0.6
Fucose	ND	–
Xylose	2.3	2.9
Glucuronic acid	ND	–
Galacturonic acid	ND	–
Mannose	6.1	6.4
Galactose	27.8	29.4
Glucose	19.8	20.9
N-acetylglucosamine	ND	–
N-acetylglucosamine	ND	–
N-acetylmannosamine	ND	–
Heptose	43.9	39.8
Total	100.4	100.0

ND, Not determined.

Table 7. Glycosyl-linkage results of CPS III following GC/MS analysis

Glycosyl residue	Percentage present
3-Linked rhamnopyranosyl (3-rha _p)	2.9
Terminally linked mannopyranosyl (t-man _p)	7.4
Terminally linked glucopyranosyl (t-glcp)	14.6
Terminally linked galactopyranosyl (t-galp)	2.2
3-Linked glucopyranosyl (3-glcp)	6.0
2- and 3-linked mannopyranosyl (2- and 3-man _p)	4.9
Terminally linked hetopyranosyl (t-hep)	23.2
3-Linked galactopyranosyl (3-galp)	8.1
4-Linked glucopyranosyl (4-glcp)	10.8
3,4-Linked heptopyranosyl (3,4-hep)	9.8
2,6-Linked glucopyranosyl (2,6-glcp)	4.2
3,7-Linked heptopyranosyl (3,7-hep)	5.9

_p, Pyranosyl.

gene products responsible for the biosynthesis of a separate capsule. CPS III was not found to contribute to the pathogenesis of *B. pseudomallei*. This capsule was not shown to be highly expressed *in vivo* by microarray analysis and was not required for virulence in the animal model. The CPS III mutant SZ1829, which contains a mutation in the BPSS1829 gene as a result of insertional inactivation, was found to be as virulent in the animal model as wild-type *B. pseudomallei*. Expression of this capsule was shown to be elevated when incubated in water but suppressed in the presence of NHS. The presence of the CPS III cluster in *B. pseudomallei* and *B. thailandensis*, both of which can survive for long periods in the environment compared with *B. mallei*, the increased expression of this capsule in water and the low level of expression of this capsule *in vivo* suggest that this capsule may contribute to the survival of *B. pseudomallei* in the environment (Aldhous, 2005; Sanford, 1995; Strauss *et al.*, 1969; Wuthiekanun *et al.*, 1995). The *wza* gene of this cluster demonstrates homology to the *amsK* gene of *Bacillus cereus*, which is involved in amylovoran biosynthesis. Amylovoran has been shown to protect the plant pathogen *Erwinia amylovora* from water stress (Bereswill & Geider, 1997).

Previous studies have demonstrated that *B. pseudomallei* produces three other CPSs in addition to CPS I and these have been structurally characterized. One is an acidic polysaccharide with the structure, [3)-2-O-acetyl- β -D-Galp-(1-4)- α -D-Galp-(1-3)- β -D-Galp-(1-5)- β -D-KDOP-(2-], which is recognized by patient sera (Nimtz *et al.*, 1997). The other two are a branched 1,4-linked glucan polymer (CP-1a) and a triple-branched heptasaccharide repeating unit composed of rhamnose, mannose, galactose, glucose and glucuronic acid (CP-2) (Kawahara *et al.*, 1998). Combined GC/MS analysis of CPS III revealed that the composition of this capsule demonstrated some similarity to the composition of the previously described capsule CP-2, also being composed of rhamnose, mannose, galactose, glucose and glucuronic acid; however, the proportions of carbohydrate residues were not similar, and the CPS III capsule was also found to contain xylose and not glucuronic acid. In addition, CPS III was determined to be composed primarily of heptose. Therefore, it is evident that the capsule identified in this study is not one of the previously described capsule structures. Some of the previously characterized capsules produced by *B. pseudomallei* have been shown to be produced under unique conditions (Kawahara *et al.*, 1998; Nimtz *et al.*, 1997). Strain variation, differences in expression of the capsules and discrepancies among purification strategies may also explain why a number of capsules have been shown to be produced by this organism. Nevertheless, the genes BPSS1825–BPSS1835 appear to be involved in the biosynthesis of a capsule with this composition. Further analysis by two-dimensional NMR would be required to establish definitively a connection between CPS III and one of the other published structures.

Recent studies have also focused on the presence of these capsule clusters in *B. pseudomallei*. Sarkar-Tyson *et al.*

(2007) identified two polysaccharide clusters, one of which corresponded to the CPS III presented in this paper, but the authors identified this cluster as type IV O-antigenic polysaccharide, which was found to be involved in virulence in a mouse model. However, a mutant in this polysaccharide did not demonstrate any difference in hydrophobicity compared with wild-type, indicating that this polysaccharide does not contribute to making the cell surface more hydrophobic, which is an advantageous characteristic for some pathogenic bacteria. The differences in virulence compared with the current work can be attributed to the use of different animal models; however, all other data seem to indicate that this capsule is not required for virulence.

B. pseudomallei is an environmental saprophyte often found in soil and stagnant water, and the incidence of infection is high in rice farmers in South-East Asia (Aldhous, 2005; Leelarasamee & Bovornkitti, 1989). This organism harbours a large genome, which explains its ability to survive for long periods of time in the environment, as well as its ability to exist as a significant pathogen in both humans and animals. The presence of multiple polysaccharide clusters in the genome and the production of multiple capsule structures under differing conditions may contribute to the ability of this organism to adapt to a variety of conditions. As demonstrated in this study, capsule expression is dependent on the particular environment, which indicates that *B. pseudomallei* produces these capsules to promote a survival advantage either in the host or in the environment. Further studies aimed at characterizing the capsules of *B. pseudomallei* will be beneficial to understand the pathogenesis of this organism and to advance further vaccine development.

ACKNOWLEDGEMENTS

This work was funded by the Canadian Institutes of Health (with a research grant (MOP-36343) to D.E.W. and by a Research Incentive grant from Athabasca University to S.L.R.-Z. Carbohydrate analysis was conducted by the Complex Carbohydrate Research Center at the University of Georgia (Athens, GA, USA) and this was supported in part by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates (Athens, GA, USA). We wish to thank the staff at the Southern Alberta Microarray Facilities (Calgary, AB, Canada) for technical support. We also thank Marina Tom for excellent technical assistance.

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